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<b>(21) International Application Number:</b> PCT/US82/01323 <b>(22) International Filing Date:</b> 27 September 1982 (27.09.82) <b>(31) Priority Application Number:</b> 306,091 <b>(32) Priority Date:</b> 28 September 1981 (28.09.81) <b>(33) Priority Country:</b> US <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 306,091 (CIP) Filed on 28 September 1981 (28.09.81) <b>(71) Applicant (for all designated States except US):</b> FRITZSCHE DODGE & OLCOTT INC. [US/US]; 76 Ninth Avenue, New York, NY 10011 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> FARBOOD, Mohamad, I. [IR/US]; 1H Meadow Lane, Princeton, NJ 08540 (US). WILLIS, Brian, J. [GB/US]; 5 Goose Cove Lane, Ramsey, NJ 07446 (US).		<b>(74) Agents:</b> MORAN, Thomas, F. et al.; Cooper, Dunham, Clark, Griffin & Moran, 30 Rockefeller Plaza, New York, NY 10112 (US). <b>(81) Designated States:</b> AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> PRODUCTION OF GAMMA-DECALACTONE  <b>(57) Abstract</b> <p>Optically active <math>\gamma</math>-decalactone is produced by culturing a microorganism capable of hydrolyzing castor oil and effecting <math>\beta</math>-oxidation of the resulting hydrolysate in the presence of castor oil or castor oil hydrolysate and a co-oxidant. The resulting <math>\gamma</math>-hydroxydecanoic acid is lactonized to form <math>\gamma</math>-decalactone.</p>		

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PRODUCTION OF GAMMA-DECALACTONEBACKGROUND OF THE INVENTION

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This invention is concerned with a microbial process for the production of optically active  $\gamma$ -decalactone. Considerable time and effort have been expended by microbiologists in the search for better processes for the production of optically active lactones. U.S. Patent 3,076,750 discloses a method of preparing certain optically active lactones and the corresponding hydroxycarboxylic acids by microbial reduction of keto-carboxylic acids. The metabolism of ricinoleic acid by some *Candida* strains was investigated by Okui et al. (J. Biochemistry, 54,536-540, 1963) who showed that  $\gamma$ -hydroxydecanoic acid was an intermediate in the oxidative degradation of ricinoleic acid. However, only trace amounts of  $\gamma$ -hydroxydecanoic acid were recovered from the fermentation medium due to the metabolism of  $\gamma$ -hydroxydecanoic acid upon completion of the fermentation, and the toxicity of ricinoleic acid to the microorganism, which limits the amount of substrate that can be used.

SUMMARY OF THE INVENTION

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This invention provides a method of producing optically active  $\gamma$ -hydroxydecanoic acid which comprises culturing or incubating a microorganism capable of hydrolyzing castor oil, and effecting  $\beta$ -oxidation of the resulting hydrolysate in the presence of castor oil, to produce  $\gamma$ -hydroxydecanoic acid.

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5 In another embodiment, the invention provides a method of producing optically active  $\gamma$ -hydroxydecanoic acid which comprises enzymatically hydrolyzing castor oil using lipase to form an enzymatic hydrolysate and culturing or incubating a microorganism capable of effecting  $\beta$ -oxidation of the enzymatic hydrolysate in the presence of said hydrolysate to produce  $\gamma$ -hydroxydecanoic acid.

10 In still another embodiment, the invention provides a method of producing optically active  $\gamma$ -hydroxydecanoic acid which comprises culturing or incubating a microorganism capable of hydrolyzing castor oil and a microorganism capable of effecting  $\beta$ -oxidation of  
15 castor oil hydrolysate in the presence of castor oil to produce  $\gamma$ -hydroxydecanoic acid.

#### DETAILED DESCRIPTION OF THE INVENTION

20 The invention provides a fermentation process for the production of optically active  $\gamma$ -hydroxydecanoic acid which may optionally be converted by lactonization to  $\gamma$ -decalactone. Depending on the embodiment of the invention employed, the fermentation process involves  
25 culturing or incubating a microorganism capable of hydrolyzing castor oil and effecting  $\beta$ -oxidation of the resulting hydrolysate, or a microorganism capable of effecting  $\beta$ -oxidation of hydrolysate of castor oil, or  
30 a microorganism capable of effecting  $\beta$ -oxidation of an enzymatic hydrolysate of castor oil, in a suitable

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medium in the presence of the castor oil or castor oil hydrolysate substrate. The use of castor oil or castor oil hydrolysate as the substrate is determined by the microorganism(s) employed in the process. A co-oxidant may be added to the culture medium in order to increase the yield of the process.

The selection of the appropriate microorganism for the process is crucial depending on the embodiment of the invention employed, the yield of product required, and the resistance to the toxicity of the fatty acids found in the castor oil hydrolysate. The microorganisms in the invention may be bacteria, yeast or filamentous fungi. Where a microorganism is employed to hydrolyze the castor oil substrate and  $\beta$ -oxidize the resulting hydrolysate, the preferred microorganisms are: Aspergillus oryzae, Candida rugosa, Geotrichum klebahnii or Yarrowia lipolytica, (formerly known as Saccharomycopsis lipolytica and previously Candida lipolytica), more preferably Yarrowia lipolytica. Where the microorganism is employed to only  $\beta$ -oxidize castor oil hydrolysate, the preferred microorganisms are: Hansenula saturnus, Candida guilliermondii, Candida albicans, Candida krusei, Candida parakrusei, Candida pseudotropicalis, Candida stellatoidea, Candida tropicalis, Aspergillus oryzae, Candida rugosa, Geotrichum klebahnii or Yarrowia lipolytica, more preferably Candida guilliermondii. Where the



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microorganism is used in combination with a lipase and with castor oil, the preferred microorganisms are: Hansenula saturnus, Candida guilliermondii, Candida albicans, Candida krusei, Candida parakrusei, Candida pseudotropicalis, Candida stellatoidea, Candida tropicalis, Aspergillus oryzae, Candida rugosa, Geotrichum klebahnii or Yarrowia lipolytica, more preferably Candida guilliermondii. Generally, any type of lipase enzyme may be used to hydrolyze the castor oil, including microbial, pancreatic, fungi or yeast.

Whereas lipase is used with the microorganism in the process of the invention, the formation of the enzymatic hydrolysate may be controlled by limiting the amount of lipase used in the process. This will avoid toxicity resulting from the presence of excessive amounts of hydrolysate. The appropriate amount of lipase required may be conveniently found by experimentation and will depend upon the lipase and microorganism used and the culturing conditions. The hydrolysis using lipase is most preferably carried out concurrently with the fermentation in the same reaction vessel. However, the hydrolysis may be carried out prior to fermentation if appropriate measures are taken to avoid the toxic effect of the hydrolysate. When castor oil is used in the invention, the concern for toxicity is eliminated because triglycerides are not toxic to the organisms. Additionally, the use of castor oil and castor oil hydrolysates as the substrate

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provide co-oxidants to the process which increase efficiency due to the presence of other fatty acids upon hydrolysis of the castor oil.

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The form in which the microorganisms are used is not critical. They can be used as the culture (suspension), i.e., including the cells and the corresponding nutrient solution, or in the form of cells suspended in a buffer solution. The cells or an enzyme extract thereof may be immobilized on a suitable solid support which may then be used to effect the transformations.

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The culture suspension is prepared by inoculation of a suitable medium with the microorganism. A suitable medium is one which contains carbon sources, nitrogen sources, inorganic salts and growth factors. Among the suitable carbon sources are for example, glucose, galactose, L-sorbose, maltose, sucrose, cellobiose, trehalose, L-arabinose, L-rhamnose, ethanol, glycerol, L-erythrithol, D-mannitol, lactose, melibiose, raffinose, meleritose, starch, D-xylose, D-sorbitol,  $\alpha$ -methyl-D-glucoside, lactic acid, citric acid and succinic acid. Among the suitable nitrogen sources are, for example, nitrogen-containing organic substances such as peptone, meat extract, yeast extract, corn steep liquor, and casein, urea, amino acids, or nitrogen

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containing inorganic compounds such as nitrates, nitriles, and inorganic ammonium salts. Among the suitable inorganic salts are, for example, phosphates, magnesium, potassium, calcium, sodium. The above mentioned  
5 nutrients in the culture medium may be supplemented with, for example, one or more vitamins of the B Group and/or one or more trace minerals such as Fe, Mo, Cu, Mn, B as desired. However, the process can be performed in a vitamin-free medium, for example, when a  
10 small amount of yeast extract is added to the medium there is no need for vitamins or trace minerals.

The cultivation of the microorganism can be carried out as a stationary culture or as a submersed culture  
15 (e.g., shaking culture, fermentors) preferably under aerobic conditions. One suitably may work in the pH range of from about 3.5 to about 8.0, and preferably in the range of from about 4.0 to about 7.5. The pH may be regulated by the addition  
20 of inorganic or organic bases, such as sodium hydroxide, potassium hydroxide, calcium hydroxide, calcium carbonate, by ion-exchange resins, or by the addition of a buffer such as phosphate or phthalate. The incubation temperature is suitably maintained at between about 15°C  
25 and about 33°C, with a range from about 20°C to about 30°C being preferred.

The process in accordance with the invention is conveniently carried out by adding castor oil or castor  
30 oil hydrolysate, as the substrate, to the culture medium at the onset of cultivation, as the sole carbon source. Alternatively, the substrate may be added in combination with another carbon source, such

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as dextrose, either during cultivation, or when cultivation is complete. The amount, level, or concentration of the substrate in the medium may vary. For example, in the case of hydrolyzed castor oil, levels of from about 0.3% to about 5% may make up the medium initially or be added during the course of the fermentation, whereas substantially any level of castor oil may be used.

The reaction time may vary depending on the composition of the culture medium and the substrate concentration. In general, shaking flask cultures require from between about 2h. and about 240h. depending upon the microorganism and the composition of the culture medium. However, when a fermentor is used the fermentation time may be reduced to about 100 h. or less.

The fermentation may be carried out using the cells of the microorganism isolated from the culture solution, or with an enzyme extract isolated from the cells in a manner known per se. In this case, the fermentation can be conveniently carried out in aqueous solution, for example in a buffer solution, in a physiological salt solution, in a fresh nutrient solution, or in water. The isolated cells or enzyme extract may be immobilized on a solid support and the desired transformation effected in the absence of the live microorganism. The transformation of the substrate may be effected by mutants of the microorganism. Such mutants can be obtained readily by methods well known in the art, for example, by exposing the cells to UV or X-rays, or customary mutagenic substances such as for example, acridine orange.



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The substrate is generally added directly to the medium. A surface-active agent or dispersion agent, such as Tween 80 (polyoxyethylenesorbitan monostearate), can also be added to an aqueous suspension of the substrate. Conventional antifoam agents, such as silicone oils (e.g., UCON), polyalkyleneglycol derivatives, maize oil, or soya oil can be used to control foaming.

The transformation of the substrate can be monitored using standard analytical techniques such as GLC, TLC, HPLC, IR and NMR. If a rapid disappearance of the substrate is observed, more substrate can then be added in order to maximize the transformation capacity of the microorganisms. The incubation is generally terminated when all the substrate has disappeared from the culture medium.

After the fermentation process is complete, the  $\gamma$ -hydroxydecanoic acid can either be lactonized in the medium to form  $\gamma$ -decalactone, or isolated and purified by conventional techniques including solvent extraction and distillation. When in situ lactonization is desired, the pH of the medium is adjusted to between about 1 and about 5, preferably between about 1 and about 3, by the addition of a suitable acid, such as hydrochloric acid, and the resulting mixture heated to between about 50°C and about 100°C, preferably between about 70°C and about 100°C for about ten minutes, depending upon the temperature, to convert the  $\gamma$ -hydroxydecanoic acid to  $\gamma$ -decalactone. The  $\gamma$ -decalactone is then recovered and purified by standard techniques. If the  $\gamma$ -hydroxydecanoic acid is recovered, it may be lactonized according to known procedures [see, for example, I.L. Finar, Organic Chemistry, 6th ed., Vol.1, p 469 (1973)].



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The following examples serve to illustrate embodiments of the invention as it is now preferred to practice it but in no way are meant to limit the scope thereof. Unless otherwise stated, weights are in grams, temperatures are in degrees centigrade and pressure in mm Hg.

EXAMPLE I

A flask containing 100 ml of 2% beef extract and 0.02% Tween 80 was autoclaved at 120°C. for 20 minutes. The medium was then inoculated with  $10^7$  cells Yarrowia lipolytica (Saccharomycopsis lipolytica)/ml of medium, and 10g of castor oil added. The culture was incubated at 26°C. on a rotary shaker (200 rpm) for one week. The pH of the medium was occasionally adjusted to 6.5-7.0. At the end of the fermentation period the pH of the medium was adjusted to 1.5 by the addition of mineral acid, and the mixture heated at 100°C. for 10 minutes. After cooling, the organic products were extracted with hexane, and hexane evaporated, and the residue distilled to provide 0.61g  $\gamma$ -decalactone having a GLC purity of 90%.

EXAMPLE II

The procedures and materials similar to those described in Example I were followed, except 0.05g decanoic acid was added each day. There was obtained 0.69g  $\gamma$ -decalactone having a GLC purity of 92%.



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EXAMPLE III

5 The procedures and materials similar to those described in Example I, except that Candida guilliermondii was used and 3g were followed of castor oil hydrolysate was added. There was obtained the desired product  $\gamma$ -decalactone in 34% yield.

EXAMPLE IV

10 By employing the procedures and materials similar to those described in Example I except that lipase is added in conjunction with castor oil, there may be obtained the desired product,  $\gamma$ -decalactone.

EXAMPLE V

15 By employing the procedures and materials similar to those described in Examples I, II and III except that other members of the genus Candida such as C. albicans, C. krusei, C. parakrusei, C. pseudotropicalis, C. stellatoidea, C. tropicalis, etc., are used, there may be obtained the desired  $\gamma$ -decalactone.

EXAMPLE VI

25 By employing the procedures and materials similar to those described in Example I, except that as a micro-organism Aspergillus oryzae is used and 3g of castor oil is added, there is obtained the desired product  $\gamma$ -decalactone (0.86g/L).

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EXAMPLE VII

By employing the procedures and materials similar to those described in Example I, except that as a micro-  
5 organism Geotrichum klebahnii is used and 3g of castor oil is added, there is obtained the desired product  $\gamma$ -decalactone (0.2g/L).

EXAMPLE VIII

10 By employing the procedures and materials similar to those described in Example I, except that as a micro-organism 'Candida guilliermondii is used and to each 100 ml of medium 100 mg of a lipase (steapsin,  
15 Nutritional Biochem Corp.) is added, the desired product  $\gamma$ -decalactone may be obtained.

The invention being thus described, it will be obvious  
20 that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention and all such modifications are intended to be included within the scope  
of the following claims:



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WHAT IS CLAIMED IS:

1. A method of producing optically active  $\gamma$ -hydroxy-decanoic acid which comprises culturing or incubating  
5 a microorganism capable of hydrolyzing castor oil and effecting  $\beta$ -oxidation of the resulting hydrolysate in the presence of castor oil to produce  $\gamma$ -hydroxy-decanoic acid.
- 10 2. A method in accordance with Claim 1 wherein said microorganism is Aspergillus oryzae, Candida rugosa, Geotrichum klebahnii or Yarrowia lipolytica.
- 15 3. A method in accordance with Claim 1 wherein the  $\gamma$ -hydroxydecanoic acid is lactonized in situ to form  $\gamma$ -decalactone and the resulting  $\gamma$ -decalactone is recovered.
- 20 4. A method in accordance with Claim 1 wherein the  $\gamma$ -hydroxydecanoic acid is recovered and lactonized to form  $\gamma$ -decalactone.
- 25 5. A method in accordance with Claim 1 wherein a co-oxidant is added to, or present during the culturing or incubating of the microorganism.
- 30 6. A method of producing optically active  $\gamma$ -hydroxy-decanoic acid which comprises culturing or incubating a microorganism capable of effecting  $\beta$ -oxidation of castor oil hydrolysate in the presence of castor oil hydrolysate to produce  $\gamma$ -hydroxydecanoic acid.

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7. A method in accordance with Claim 6 wherein said microorganism is Hansenula saturnus,  
Candida guilliermondii, Candida albicans, Candida krusei, Candida parakrusei, Candida pseudotropicalis,  
Candida stellatoidea, Candida tropicalis, Aspergillus oryzae, Candida rugosa, Geotrichum klebahnii or  
Yarrowia lipolytica.

8. A method in accordance with Claim 6 wherein the  $\gamma$ -hydroxydecanoic acid is lactonized in situ to form  $\gamma$ -decalactone and the resulting  $\gamma$ -decalactone is recovered.

9. A method in accordance with Claim 6 wherein the  $\gamma$ -hydroxydecanoic acid is recovered and lactonized to form  $\gamma$ -decalactone.

10. A method in accordance with Claim 6 wherein a co-oxidant is added to, or present during the culturing or incubating of the microorganism.

11. A method of producing optically active  $\gamma$ -hydroxydecanoic acid which comprises enzymatically hydrolyzing castor oil using lipase to form an enzymatic hydrolysate and culturing or incubating a microorganism capable of effecting  $\beta$ -oxidation of the enzymatic hydrolysate in the presence of said hydrolysate to produce  $\gamma$ -hydroxydecanoic acid.



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12. A method in accordance with Claim 11 wherein said microorganism is Hansenula saturnus, Candida guilliermondii, Candida albicans, Candida krusei, Candida parakrusei, Candida pseudotropicalis, Candida stellatoidea, Candida tropicalis, Aspergillus oryzae, Candida rugosa, Geotrichum klebahnii or Yarrowia lipolytica.
13. A method in accordance with Claim 11 in which the lipase enzyme is microbial, pancreatic, fungi or yeast.
14. A method in accordance with Claim 11 wherein the  $\gamma$ -hydroxydecanoic acid is lactonized in situ to form  $\gamma$ -decalactone and the resulting  $\gamma$ -decalactone is recovered.
15. A method in accordance with Claim 11 wherein the  $\gamma$ -hydroxydecanoic acid is recovered and lactonized to form  $\gamma$ -decalactone.
16. A method in accordance with Claim 11 wherein a co-oxidant is added to, or present during the culturing or incubating of the microorganism.
17. A method of producing optically active  $\gamma$ -hydroxydecanoic acid which comprises fermenting Yarrowia lipolytica in the presence of castor oil and a co-oxidant to produce  $\gamma$ -hydroxydecanoic acid and lactonizing the  $\gamma$ -hydroxydecanoic acid in situ to form  $\gamma$ -decalactone by adjusting the pH of the fermentation media and recovering said  $\gamma$ -decalactone.





# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US82/01323

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all)		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC- C12P 7/42, C12P 17/04 US CL- 435/126, 435/146		
<b>II. FIELDS SEARCHED</b>		
Classification System      Classification Symbols		
U.S.	435/123, 126, 136, 146, 911, 918, 921, 922, 923, 924, 930	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched:		
CHEMICAL ABSTRACTS, VOL. 56-95 (1962-1981)		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages **	Relevant to Claim No. 1 <sup>3</sup>
Y, A	N, THE JOURNAL OF BIOCHEMISTRY, 54 (6), ISSUED 1963, SEIICHI OKUI ET AL, METABOLISM OF HYDROXY FATTY ACIDS, 536-540.	1-17
Y	N, BAILEY'S INDUSTRIAL OIL AND FAT PRODUCTS, VOL. 1, FOURTH EDITION, ISSUED 1979, DANIEL SWERN, JOHN WILEY & SONS, NEW YORK, SEE PAGES 453-456.	6-16
A	US, A, 3,076,750 (MVIS) 05 FEBRUARY 1963.	1-17
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<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search: 27 DECEMBER 1982		Date of Mailing of this International Search Report: 30 DEC 1982
International Searching Authority: ISA/US		Signature of Authorizing Officer: Esther M. Kepplinger